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Core sequence of ATP regulatory module in receptor guanylate cyclases

Teresa Duda, Rafal M. Goraczniak and Rameshwar K. Sharma

The Unit of Regulatory and Molecular Biology, Pennsylvania College of Optometry, 1200 West Godfrey Avenue, Philadelphia, PA 19141, USA

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Atrial natriuretic factor (ANF) and C-type natriuretic peptide (CNP)-activated guanylate cyclases are single-chain transmembrane-spanning proteins, containing both ligand binding and catalytic activities. In both proteins, ligand binding to the extracellular receptor domain activates the cytosolic catalytic domain, generating the second messenger cyclic GMP. Studies with ANF receptor guanylate cyclase (ANF-RGC) have indicated that obligatory in this activation process is an ATP-dependent step. ATP directly binds to the cyclase and bridges the events of ligand binding and signal transduction. A defined ATP-regulated module (ARM) sequence (Gly^{503}-Arg-Gly-Ser-Asn-Tyr-Gly^{509}) in the cyclase is critical in the ATP-mediated event. Through genetic remodeling techniques, we have now identified the core ARM sequence that is essential in both ANF and CNP signaling. This sequence is Gly-Xa-Xa-Xa-Gly, represented by Gly^{505}-Ser-Asn-Tyr-Gly^{509} in the case of ANF-RGC ARM and by Gly^{499}-Ser-Ser-Tyr-Gly^{503} in the CNP receptor guanylate cyclase ARM.

Guanylate cyclase; ATP-regulatory module; Atrial natriuretic factor receptor; Type C natriuretic peptide receptor

1. INTRODUCTION

One important class of guanylate cyclases is that its members are also cell surface receptors of natriuretic factors. Two such guanylate cyclases have been characterized. One is the atrial natriuretic factor (ANF) receptor (ANF-RGC) [1–7], and the other is a receptor for type C natriuretic peptide (CNP-RGC) [8]. ANF and CNP are structurally related peptide hormones that regulate hemodynamics of the physiological processes of diuresis, water balance, and blood pressure [8–10]; a third related hormone is the type B natriuretic peptide (BNP) [11], but its receptor is not yet identified. One important second messenger of these hormones is cyclic GMP (reviewed in [12–14]).

ANF and CNP signal through their respective receptor guanylate cyclases, ANF-RGC and CNP-RGC. Both of these receptors show sequence similarity, and both contain a single membrane-spanning helical domain which divides the protein into two roughly equal portions, the N-terminal extracellular and the C-terminal intracellular. The receptor domain lies in the extracellular portion and the intracellular portion contains two domains, the one adjacent to the transmembrane is termed 'kinase-like' domain due to its sequence similarity to the tyrosine kinase family, and the C-terminal region contains the catalytic domain [1,15]. In both proteins ligand binding to the extracellular receptor domain activates the cytosolic catalytic domain, generating the second messenger cyclic GMP [2,16].

Studies with ANF-RGC have indicated that obligatory in this activation process is an intervening step, which is regulated by ATP [17,18]. ATP binding causes an allosteric change in guanylate cyclase, bringing it to the activated catalytic state. A defined ATP-regulated module (ARM) of ANF-RGC with a sequence of Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} is critical in ATP binding and amplification of the hormonal signal [4,19]. CNP-RGC contains a structural motif (Gly^{499}-Xa-Xa-Xa-Gly^{503}) which is shared by a section of the ANF-RGC ARM sequence. This raises an intriguing question: is this the sequence through which ATP amplifies both ANF and CNP signals?

If the answer is yes, it would indicate two things: (i) that this is the core ARM sequence which is critical in ATP binding and in amplification of the ANF signal; and (ii) that this core ARM sequence, in an identical fashion, amplifies the CNP signal. This will then provide a unified mechanism by which ATP amplifies both ANF and CNP signals. The present study addresses these issues.

2. MATERIALS AND METHODS

The 5-kDa CNP used in these studies was a 45 amino-acid peptide, Ser-Gln-Asp-Ser-Ala-Phe-Arg-Ile-Gln-Glu-Arg-Leu-Arg-Asn-Ser-Lys-Met-Ala-His-Ser-Ser-Ser-Cys-Phe-Gly-Gln-Lys-Ile-Asp-Arg-Ile-Gly-Ala-Val-Ser-Arg-Leu-Gly-Cys-Asp-Gly-Leu-Arg-Leu-Phe; and ANF (rat, residues 8–33) was a 26 amino-acid peptide, Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr. These peptides were purchased from Peninsula Laboratories; GTP, cyclic GMP and bovine serum albumin

Correspondence address: R.K. Sharma, Unit of Regulatory and Molecular Biology, Pennsylvania College of Optometry, 1200 West Godfrey Avenue, Philadelphia, PA 19141, USA. Fax: (1) (215) 276-6081.

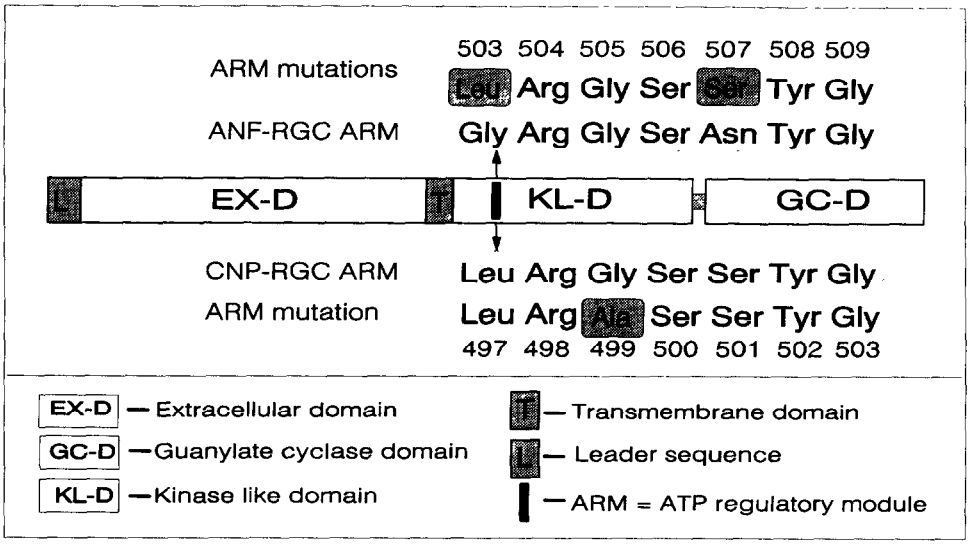

Fig. 1. Representation of GCα-Dmut (ANF-RGC) and CNP-RGC mutants. The mutated amino acid residues corresponding to the ARMs of ANF-RGC and CNP-RGC are indicated by shaded boxes. The appropriate changes in amino acid residues were created by site-directed mutagenesis as described in section 2.

were from Sigma; ATPγS and ATP were from Boehringer-Mannheim; [^{125}I]NaI was from Amersham; cell culture media were from Gibco and restriction enzymes were purchased from USB and BioLabs.

The CNP-RGC cDNA clone was isolated from a human retina cDNA library (Duda, T., Goraczniak, R., Sitaramayya, A. and Sharma, R.K., manuscript submitted); ANF-RGC (GCα-Dmut) was constructed from GCα cDNA as described previously [2]. Mutants corresponding to the ARM sequences of ANF-RGC and CNP-RGC were constructed as schematically represented in Fig. 1 and are described below.

2.1. *GCα-DmutLeu^{503}Ser^{507} (GCα-Dmut/CNP-RGC ARM)*

The 1.9 kb *SalI–XbaI* fragment of GCα cDNA was subcloned into the pSelect-1 vector; Gly^{503} was changed to Leu and Asn^{507} to Ser, using the mutagenic primer 5′-CAGGGAGCCATAACTG-GAGCCTCGCAGACTCAGGGTCAG-3′, and the selection-ampicillin-repair primer (Promega mutagenesis kit). The *EcoRV–XbaI* fragments of GCα-Dmut cDNA in the pBluescript vector were replaced with the *EcoRV–XbaI* fragment excised from a pSelect-cDNA recombinant.

2.2. *CNP-RGCAla^{499}*

Mutagenesis leading to the change of Gly^{499} into Ala was performed on the 1.85 kb *SalI–XbaI* fragment of CNP-RGC cDNA subcloned into pSelect-1 vector using mutagenic, 5′-GCCGTAACTG-GAGGCCCGCAGCGACAG-3′ and the selection-ampicillin-repair primers. The 0.7 kb *EcoRV–XbaI* fragment excised from pSelect-1 cDNA recombinant replaced the *EcoRV–XbaI* fragment of CNP-RGC cDNA.

All the mutated recombinants were sequenced [20] to confirm their identities and correct ligations. The mutated-GCα and CNP-RGC cDNAs were individually subcloned into the *XhoI–SmaI* site of the pSVL vector to create pSVL-mutated cDNA expression constructs.

For expression studies, COS-7 cells (simian virus 40 (SV40)-transformed African green monkey kidney cells) were transfected with the expression vector by the calcium phosphate technique [21]; 60 h after transfection, cells were washed with 50 mM Tris-HCl (pH 7.5) /10 mM $MgCl_2$ buffer, scraped into 2 ml of ice-cold buffer, homogenized, centrifuged for 15 min at 5,000 × *g* and washed with the same buffer. The pellet represented the crude membranes. In control experiments, the crude membranes prepared from cells transfected with the pSVL vector alone were used. The crude membranes were assayed for guanylate cyclase activity [6]. The ATP binding was assayed as in [19].

3. RESULTS AND DISCUSSION

In the present study we have extended the genetic remodeling technique to determine the role of the ARM core structural motif Gly-Xa-Xa-Xa-Gly in ANF and CNP signaling. Previous studies with ANF-RGC have shown that ARM sequence Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} positioned at 40 amino acid residues distal to the last carboxy-terminal amino acid residue of the transmembrane domain is essential in the ATP-mediated occurrence that bridges the events of ANF binding and signal transduction [19]. The counterpart of this ANF-RGC ARM structural motif in CNP-RGC is Xa^{497}-Xa-Gly^{499}-Xa-Xa-Xa-Gly^{503}. This indicates that there is a change in the sequence form – there being two glycine-surrounded domains (Gly-Xa-Gly-Xa-Xa-Xa-Gly) in ANF-RGC and only one (Gly-Xa-Xa-Xa-Gly) in CNP-RGC.

To determine whether the CNP-RGC ARM sequence could substitute the ANF-RGC ARM sequence in ANF signaling, in the first step GCα was genetically tailored to create a mutant, GCα-Dmut, which is structurally and functionally identical to the cloned wild-type ANF-RGC [2]. In the second step, ARM sequence of the genetically constructed ANF-RGC – Gly^{503}-Arg-Gly-Ser-Asn^{507}-Tyr-Gly^{509} – was changed to that of the CNP-RGC ARM (GCα-Dmut/CNP-RGC ARM mutant) – Leu-Arg-Gly-Ser-Ser-Tyr-Gly – i.e. Gly^{503} was converted to Leu and Asn^{507} to Ser (Fig. 1). The coding sequence of the mutated protein was introduced into an

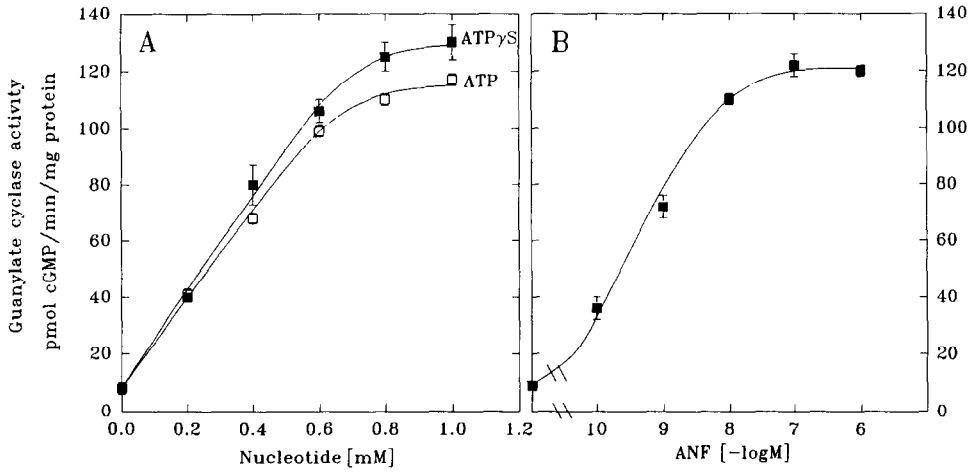

Fig. 2. Effect of ATP (or ATPγS) and ANF on guanylate cyclase activity. Membranes of Cos7 cells transfected with GCα-DmutLeu^{503}Ser^{507} (GCα-Dmut/CNP-RGC ARM), as described in section 2, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor [18] in the presence of (A) ANF (0.1 μM) with indicated additions of ATP (or ATPγS) and (B) in the presence of 0.8 mM ATP with varied additions of ANF. The experiments were done in triplicate and repeated three times, although the data depicted are from one typical experiment; means ± S.D. are shown.

expression vector, pSVL, under the transcriptional control of the SV40 late promoter, which was then used to transfect COS-7 cells. The particulate fractions of these cells were appropriately treated and analyzed for the cyclase and ATP-binding activities.

Because earlier studies with ANF-RGC have indicated that maximal guanylate cyclase activity is attained with 0.1 μM ANF in the presence of 800 μM ATP [4,18], we first used the same concentrations of the above reagents to assess the basal and the ANF-stimulated cyclase activities of the mutated protein. The plasma membranes containing the mutated-protein showed 30- to 40-fold higher basal activity than the membranes of control cells transfected with pSVL alone (Table I), indicating that the encoded protein is a guanylate cyclase.

To determine the role of CNP-RGC ARM sequence in ATP-mediated ANF signaling, the membranes of the transfected cells were incubated with a series of concentrations of ATP, or ATPγS, in the presence of ANF (0.1 μM). Both ATP and ATPγS stimulated the cyclase activity in a dose-dependent fashion; the maximal activity was observed at ~800 μM and the half-maximal activation (EC_{50}) occurred between 300 and 400 μM (Fig. 2A).

Neither ATP nor its non-hydrolyzable analog, ATPγS, by itself altered the basal cyclase activity of the CNP-RGC ARM-mutant; similarly, ANF alone had only marginal effect on the basal cyclase activity (Table I).

To determine the kinetics of ANF-dependent activation of guanylate cyclase, membranes of the cells transfected with GCα-Dmut/CNP-RGC ARM mutant cDNA were incubated with different concentrations of

Table I

Guanylate cyclase activity in membranes of transfected COS-7 cells

Transfection	Guanylate cyclase activity (pmol cyclic GMP/min/mg protein)				
	Basal	+ANF (10^{-7} M)	+CNP (10^{-7} M)	+ATP (0.8 mM)	+ATPγS (0.8 mM)
pSVL (control)	~0.1	~0.1	~0.1	~0.1	~0.1
GCα-Dmut	7.0 ± 1.0	17.0 ± 2.1	8.0 ± 0.9	7.0 ± 1.0	7.0 ± 0.9
GCα-DmutVal^{505}Asn^{506}	5.0 ± 0.4	9.1 ± 1.0	5.4 ± 0.6	5.1 ± 0.6	5.2 ± 0.6
GCα-DmutLeu^{503}Ser^{507}	8.1 ± 0.5	18.0 ± 1.2	9.2 ± 1.1	9.1 ± 1.0	8.9 ± 0.9
CNP-RGC	12.1 ± 0.9	12.5 ± 1.0	15.0 ± 1.5	12.0 ± 1.5	12.2 ± 1.3
CNP-RGCAla^{499}	15.4 ± 1.0	14.0 ± 1.5	13.5 ± 1.5	14.0 ± 1.5	14.2 ± 1.5

COS-7 cells were transfected with appropriate GCα or CNP-RGC mutant cDNAs in a pSVL expression vector. Membranes were prepared as described in section 2, and guanylate cyclase activity was determined [18]. The experiments were done in triplicate and repeated three times, although the data given are from one typical experiment.

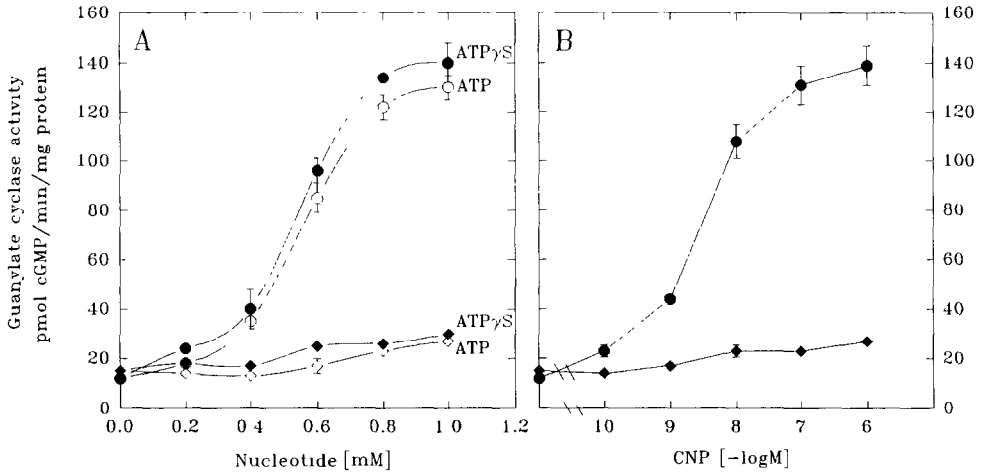

Fig. 3. Effect of ATP (or ATPγS) and CNP on guanylate cyclase activity. Membranes of Cos7 cells transfected with CNP-RGC or CNP-RGCAla^{499}, as described in section 2, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor [18] in the presence of (A) CNP (0.1 μM) with indicated additions of ATP (or ATPγS) and (B) in the presence of 0.8 mM ATP with varied additions of CNP. The experiments were done in triplicate and repeated twice; means ± S.D. are shown. Solid and open circles represent the membranes expressing wild-type CNP-RGC and the squares represent the CNP-RGCAla^{499} mutant.

ANF in the presence of saturating amounts of ATP (800 μM). ANF stimulated the guanylate cyclase activity in a dose-dependent fashion (Fig. 2B); the EC_{50} concentration was 5 nM, an excess of threefold stimulation occurring well below 10^{-10} M (Fig. 2B).

To verify that the glycine cluster sequence Gly-Xa-Xa-Xa-Gly is directly involved in the ATP effect, the GCα-Dmut/CNP-RGC ARM mutant was scrutinized for its ATP-binding activity. Comparison of [α-^{32}P]ATP binding by membranes expressing the Gly-Xa-Xa-Xa-Gly sequence with those expressing the disrupted glycine sequence mutant, Gly-Xa-Xa-Xa-Xa-Xa-Gly (GCα-DmutVal^{505},Asn^{506}, [19]), showed that the GCα-Dmut/CNP-RGC ARM mutant had over 2-fold higher ATP-binding activity than the glycine-disrupted mutant (Table II).

Table II

ATP binding to membranes of transfected COS-7 cells

Transfection	Specific ATP binding (cpm/mg protein)
pSVL (control)	30,314 ± 500
GCα-Dmut	106,930 ± 1,900
GCα-DmutVal^{505}Asn^{506}	48,833 ± 1,150
GCα-DmutLeu^{503}Ser^{507}	102,717 ± 1,200
CNP-RGC	111,021 ± 1,500
CNP-RGCAla^{499}	47,078 ± 950

Membranes of COS-7 cells were incubated in a total volume of 100 μl with [α-^{32}P]ATP (6×10^5 cpm/tube; specific activity 3,000 Ci/mmol) in the presence of 4 mM Mg^{2+} at room temperature for 10 min. The membranes were filtered through GF/C filters and washed four times with ice-cold phosphate-buffered saline, pH 7.5. Non-specific binding was measured in the presence of 1 mM ATP. Specific binding was calculated by subtracting the non-specific radioactivity bound to the filters. Values are means of duplicate determinations. Experiments were repeated twice, and the values were normalized to depict a typical experiment.

These results are virtually identical to those previously reported for the ANF-RGC which contains the wild-type ARM sequence [19], indicating that the CNP-RGC ARM sequence form, Gly-Xa-Xa-Xa-Gly, is able to effectively substitute the ANF-RGC ARM sequence and that the presence of both ATP and ANF is required for ANF signaling. These findings also indicate that in the originally identified ANF-RGC ARM sequence, Gly^{503}-Xa-Gly^{505}-Ser-Xa-Tyr-Gly^{509} [2], the residues Gly^{503}-Xa- constituting the first glycine domain (Gly^{503}-Xa-Gly^{505}) are non-essential in ANF signaling, and suggest that the second glycine cluster sequence Gly^{505}-Ser-Xa-Tyr-Gly^{509} meets the requirement of being the ATP regulatory module of guanylate cyclase that is critical in both binding and potentiating the ATP-mediated ANF signaling event.

These results emphasizing the importance of core ARM sequence in ANF signaling raise a related question: could this sequence form – Gly^{499}-Xa-Xa-Xa-Gly^{503} – also bridge the CNP-binding and catalytic guanylate cyclase signal transduction events?

To answer this question, ATP studies were conducted with CNP-RGC and its mutant in which the glycine cluster was disrupted by changing the Gly^{499}-Xa-Xa-Xa-Gly^{503} sequence to Ala^{499}-Xa-Xa-Xa-Gly^{503}, i.e. Gly^{499} was changed to Ala by site-directed mutagenesis. Ala was the choice amino acid because both Ala and Gly are neutral and similar in size and thus the substitution in these amino acids would cause minimum deviation in the tertiary structure of the protein (Fig. 1). The coding sequences of CNP-RGC and the mutated protein were introduced into expression vectors, which were then used to transfect the COS-7 cells (vide supra).

The particulate fractions of these cells were appropriately treated and analyzed for the cyclase and ATP-binding activities.

The plasma membranes of the wild-type CNP-RGC cells (a) showed 80-fold higher basal activity than the membranes of cells transfected with pSVL alone, demonstrating that the encoded protein is a guanylate cyclase (Table I); (b) did not respond to either ATP (800 μM) or CNP (0.1 μM) alone in stimulating the basal cyclase activity, indicating that these agents by themselves are unable to potentiate the cyclase activity (Table I); (c) in the presence of saturating amounts of CNP (0.1 μM), responded to ATP (or ATPγS) stimulation of guanylate cyclase activity in a dose-dependent fashion with the maximal cyclase response occurring at ~800 μM ATP and the half-maximal activation (EC_{50}) at ~500 μM ATP (Fig. 3A); (d) in the presence of ATP (800 μM), showed a dose-dependent stimulation of guanylate cyclase activity by CNP, the EC_{50} concentration was below 5 nM, an excess of twofold stimulation occurring well below 10^{-10} M, and a 14-fold cyclase stimulation occurring at 0.1 μM CNP (Fig. 3B), and under identical conditions, ANF had no effect on CNP signaling.

In contrast to the above results, the CNP-RGC-mutant with the disrupted glycine cluster did not significantly respond in its cyclase activity to any tested concentration of ATP (or ATPγS) in the presence of saturating amounts of CNP (Fig. 3A) and, likewise, showed no stimulation of cyclase activity in response to the CNP concentrations up to 0.1 μM (Fig. 3B). The basal cyclase activity of both the wild type and the mutant were almost the same, however. This finding establishes the obligatory role of glycine cluster sequence Gly^{499}-Xa-Xa-Xa-Gly^{503} in ATP-regulated CNP signaling. This sequence, however, has no effect on the basal cyclase activity.

To verify the role of the glycine cluster sequence in defining the ATP-binding site, the wild-type CNP-RGC and its mutant were scrutinized for their ATP binding activities (Table II). The wild-type receptor showed almost 4-fold higher binding activity over the control cells expressing the pSVL expression vector, but the mutant with the disrupted glycine cluster had only marginally higher ATP binding activity above the control cells. These results prove that the sequence Gly^{499}-Xa-Xa-Xa-Gly^{503} of CNP-RGC is critical in both ATP binding and potentiating the cyclase activity. This sequence, therefore, defines the ATP-regulatory module (ARM) of CNP-RGC, which bridges the steps of ligand binding and signal transduction.

These studies do not prove that the Gly-Xa-Xa-Xa-Gly motif is the direct ATP binding site. But a 'predicted nucleotide-binding protein model' indicates that the second glycine in the structural motif Gly-Xa-Gly-Xa-Xa-Gly is crucial in the direct GTP binding to the p^{21} protein [22]. This suggests a parallel between the structural motifs of GTP-binding proteins and the ATP-binding receptor guanylate cyclases. It is, therefore, a good possibility that the middle glycine in the ARM sequence – Gly-Xa-Gly-Xa-Xa-Gly – is the direct binding site of ATP. As has been suggested for the 'nucleotide-binding protein model' [22], it does not mean that the modification of the middle glycine will result in the complete elimination of the nucleotide binding to the protein. Such a change may merely result in the 'altered mode of binding of the nucleotide' [22]. This interpretation fits our findings where we observe that disruption of the middle glycine cluster does not result in the complete elimination of ATP binding with the ANF-RGC and CNP-RGC mutants (Table II).

In conclusion, we have identified the core ARM sequence of ANF-RGC and CNP-RGC that is essential in the ATP binding and the ANF-dependent and CNP-dependent cyclase activities. This finding provides a unified mechanism for the operation of both ANF and CNP signaling processes, and supports the multi-module concept of guanylate cyclase transduction system [19].

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